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Action of metformin on erythrocyte membrane fluidity in vitro and in vivo

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Abstract

The lipid domains of the cell membrane are believed to be one of the sites where biguanides exert their antihyperglycemic effect. We have examined the effects of metformin on the membrane fluidity of intact erythrocytes in vivo and in vitro. Membrane fluidity was measured by monitoring changes in the anisotropy of the fluorescent probe 6-antroyloxystearic acid (6-AS). The erythrocyte membranes from patients with non-insulin dependent diabetes mellitus treated with metformin were more fluid than those from non-insulin dependent diabetes mellitus patients treated by diet or healthy controls. There was no correlation between membrane fluidity and the plasma lipids or the parameters of metabolic control, suggesting that the high fluidity is an effect of metformin itself. Incubation of erythrocytes from healthy controls and diabetic patients treated by diet or glibenclamide with metformin in vitro confirmed that metformin increases the fluidity of erythrocyte membranes. In vitro metformin did not alter the fluidity of membranes from diabetic patients treated with metformin, perhaps because the basal high fluidity due to their in vivo interaction with plasma metformin could be increased no further. Since insulin appears to be required for the antihyperglycemic effect of metformin, the effect of insulin on membrane fluidity was also evaluated. Insulin generally had a small fluidizing effect on erythrocytes in vitro. The fluidizing action of both insulin and metformin could represent a membrane event common to the hormone and drug leading to additive or synergistic effects in vivo. © 1997 Elsevier Science B.V.

Keywords: Diabetes mellitus, non-insulin dependent; Metformin; Insulin; Erythrocyte membrane; Lipid fluidity

1. Introduction

The biguanide metformin is an antihyperglycemic agent that is widely used as an oral treatment of patients with non-insulin dependent diabetes mellitus. Its mechanism of action appears to be very complex (Bailey, 1992; Bailey, 1993; Dunn and Peters, 1995; Wiernsperger, 1996). It decreases intestinal glucose use and gluconeogenesis without changing the plasma lactate concentration or the rate of plasma lactate turnover (Stumwoll et al., 1995) and improves glucose use by muscle and adipose tissues (Matthaei and Greten, 1991; Matthaei et al., 1992).

The sites at which metformin acts have not yet been identified. Matthaei et al. (1993) found no specific biguanide receptors in the plasma membrane of isolated adipocytes. Other investigators have studied the behaviour of [^{14}C]metformin linked with azidophenylglyoxal with isolated red cell membranes. The highest radioactivity was found in the range of protein band 4.2–4.5 where there is the erythrocyte glucose transporter (Freisleben et al., 1996). Schäfer (1983) has proposed that the biguanides act on the lipid domains of membranes which can regulate the activity of membrane proteins (Hagve, 1988) by altering their diffusion properties i.e. membrane fluidity. Thus, metformin could act rather non-specifically (Schäfer, 1983), as the biguanide group is a strong base which is completely protonated at physiological pH. Biguanides could introduce positive charges which decrease the negative charge at the membrane surface and it has been suggested that

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thereby these drugs might make the membrane more rigid (Schäfer, 1976). However, Freisleben et al. (1992) examined the effect of low concentrations of metformin (0.5 μM) on lyophilized erythrocyte membranes using electron paramagnetic resonance and found an increase in membrane fluidity. Similar results have also been obtained using artificial membranes (Wiernsperger, 1996).

Erythrocytes are an interesting model for studying the action of metformin as they are one of the most glucose-consuming tissues. Glucose consumption and/or metabolism by erythrocytes is decreased in insulin and non insulin dependent diabetes mellitus (Kato et al., 1990; Civelek et al., 1991; Conget et al., 1991; Donatelli et al., 1991; Yoa et al., 1993). Metformin stimulates the uptake of glucose in rat erythrocytes in vitro and insulin enhances the effect of metformin, although erythrocytes are not insulin-sensitive cells (Wiernsperger and Rapin, 1995). Insulin also appears to be required for metformin to stimulate glucose uptake by other cells (Wollen and Bailey, 1988; Wiernsperger, 1996).

Although these data were obtained under a variety of conditions, they suggest that a membrane event common to insulin and metformin could regulate glucose uptake. Membrane fluidity appears to be a good candidate, since insulin also modifies membrane fluidity (Bryszewska and Leyko, 1983; Dutta-Roy et al., 1985; Santini et al., 1992) and that glucose uptake is correlated with an increase in lipid fluidity following the addition of fluidizing fatty acids (More and Jones, 1983).

We have therefore studied the in vitro effect of metformin on the fluidity of erythrocyte membrane from both healthy subjects and patients with non insulin dependent diabetes mellitus treated by either diet, metformin and/or sulfonylurea glibenclamide. The influence of a hyperinsulinemic medium was also examined in vitro to study the insulin–metformin interactions. Lastly, erythrocytes from the patients treated with metformin or metformin plus glibenclamide were used to study the in vivo effect of metformin on erythrocyte membrane fluidity.

2. Subjects and methods

2.1. Subjects

The patients with non-insulin dependent diabetes mellitus taking part in the study were 32 Caucasian men under various treatments. 10 were treated by diet alone. They were taught a weight reducing diet divided in three main meals according to their food habits assessed by a seven-day recall. 6 diabetic patients were treated by glibenclamide (10–15 mg daily), 9 by metformin (850 mg twice daily) and 7 by metformin (850 mg twice daily) plus glibenclamide (15 mg daily). The controls were 19 male volunteers without any family history of diabetes or hyperlipidemia, on no regular medication and without any metabolic abnormalities. The clinical characteristics of healthy subjects and patients, their blood glucose control and plasma lipid profiles are shown in Table 1. No subject received any drug other than the antidiabetic, that could influence membrane fluidity (Goldstein, 1984). Alcoholics were excluded from the study, as alcohol alters membrane fluidity (Hrelia et al., 1986). Written informed consent was obtained from each subject and the study protocol was approved by the regional Ethics Committee.

2.2. Collection of blood samples

Blood samples were taken from an antecubital vein without venous stasis of subjects fasted for 12 h, into tubes containing EDTA.

Table 1
Clinical and biological characteristics of healthy subjects and patients with non insulin dependent diabetes mellitus

	Healthy subjects ($n = 19$)	Diabetic patients treated by				
		diet ($n = 10$)	glibenclamide ($n = 6$)	metformin ($n = 9$)	Metf + Glib ($n = 7$)	ANOVA (P)
Age (years)	41.5 ± 1.5	48.5 ± 3.3 [a]	56.2 ± 2.9 [b]	56.4 ± 2.6 [c]	53.1 ± 2.6 [b]	P < 0.0001
Diabetes duration (years)	—	6.2 ± 2.1	3.8 ± 0.8	6.2 ± 3.3	8.1 ± 3.7	
Body mass index (kg/m^2)	25.0 ± 0.8	32.1 ± 2.0 [b,f]	26.0 ± 1.4	29.0 ± 1.4 [b]	31.3 ± 1.6 [b,f]	$P < 0.001$
Fasting blood glucose (mmol/l)	5.1 ± 0.4	8.3 ± 0.7 [d]	10.0 ± 0.8 [d]	10.0 ± 0.9 [e]	12.2 ± 1.1 [e,g]	$P < 0.0001$
Hemoglobin A1c (%)	5.3 ± 0.1	6.9 ± 0.4 [c]	6.8 ± 0.8 [c]	6.5 ± 0.2 [c]	7.2 ± 0.5 [d]	$P < 0.0001$
Total cholesterol (mmol/l)	5.73 ± 0.20	5.40 ± 0.27	5.85 ± 0.43	5.51 ± 0.20	5.35 ± 0.21	NS
Total triglycerides (mmol/l)	1.35 ± 0.08	1.51 ± 0.16	2.12 ± 0.27	1.76 ± 0.37	1.83 ± 0.36	NS
Cholesterol/triglygerides ratio	4.51 ± 0.28	3.98 ± 0.47	3.20 ± 0.75	4.54 ± 0.93	3.91 ± 0.99	NS
HDL cholesterol (mmol/l)	1.35 ± 0.20	1.34 ± 0.13	1.42 ± 0.10	1.31 ± 0.10	1.24 ± 0.10	NS
LDL cholesterol (mmol/l)	3.35 ± 0.15	3.37 ± 0.23	3.32 ± 0.46	3.45 ± 0.30	3.02 ± 0.31	NS

Data are presented as the mean ± standard error (S.E.). NS: not significant; HDL: high-density lipoproteins; LDL: low-density lipoproteins; ANOVA: analysis of variance.
[a] $P < 0.05$, [b] $P < 0.005$, [c] $P < 0.001$, [d] $P < 0.0005$, [e] $P < 0.0001$ versus healthy subjects; [f] $P < 0.05$ versus glibenclamide treated patients; [g] $P < 0.05$ versus diet treated patients.

2.3. Laboratory methods

Plasma glucose was measured by the glucose oxidase method with a Beckman BGA II Glucose Analyser (Beckman Instruments, Fullerton, CA, USA). Hemoglobin A1c was measured by high-performance liquid chromatography with an ion exchange column (Bio-Rad, Paris, France). Plasma triglyceride and cholesterol were measured enzymatically (Boehringer-Mannheim, Mannheim, Germany) on a Prisma autoanalyzer (Wiman, Stockolm, Sweden). High-density lipoprotein cholesterol was measured in plasma after precipitation of very-low-density and low-density lipoproteins with phosphotungstic acid. Low-density lipoprotein cholesterol was estimated using the Friedewald equation (Friedewald et al., 1972).

2.4. Fluorescent probes

The fluorescent probe 6-(9-anthroyloxy)stearic acid was purchased from Molecular Probes (Eugene, OR, USA). Stock solutions were prepared in ethanol (2×10^{-3} M) and kept in the dark at -20°C.

2.5. Erythrocyte labelling

Whole blood (0.5 ml) was centrifuged for 10 min at $300 \times g$ and the plasma discarded. The erythrocytes were washed twice in a phosphate-buffered saline (PBS) containing 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.4 mM KH_2PO_4 and 5.5 mM glucose, pH 7.2. The washed erythrocytes were resuspended in PBS and labelled by adding 3 μl probe solution to 3 ml erythrocyte suspension (5×10^6 cells/ml; final probe concentration: 2×10^{-6} M) and incubating the mixture for 30 min at 37°C. Free probe molecules were removed by washing the cells in PBS. The cells were finally resuspended in 300 μl medium for the in vitro metformin and/or insulin tests.

2.6. In vitro interaction of erythrocytes with metformin

Labelled erythrocytes were resuspended in PBS or PBS containing insulin (30 μU/ml: Actrapid HM, Novo Nordisk Laboratories, Roskilde, Denmark), with or without metformin (Lipha, Lyon, France). The final concentration of metformin was 7×10^{-8} M. This concentration was chosen to provide a metformin/red cell ratio nearly similar to that obtained in vivo after a single therapeutic dose of metformin. Erythrocyte suspensions were incubated for 30 min at 37°C, diluted with 2.7 ml incubation medium and immediately processed for anisotropy measurements without further washing. Thus, fluorescence measurements were made on the cells while they remained in the incubation medium.

2.7. Fluorescence determinations

Membrane fluidity was measured by steady-state fluorescence polarization, using a continuous excitation instrument (Fluofluidimeter, Regulest, Florange, Moselle, France). The fluorescence of an unlabelled erythrocyte suspension (blank) was measured before each series of experiments to evaluate the corrections for light scattering. The vertical $Ib_{\parallel}$ and $Ib_{\perp}$ components for the blank were measured, followed by the corresponding fluorescence intensities $If_{\parallel}$ and $If_{\perp}$ of the labelled sample. Fluorescence anisotropy $\langle r \rangle$, which is inversely proportional to membrane fluidity was determined from the following relationship:

$$\langle r \rangle = \frac{(If\| - Ib\|) - (If_{\perp} - Ib\perp)}{(If\| - Ib\|) + 2(If_{\perp} - Ib_{\perp})}$$

The intra-assay coefficient of variation (5 determinations on the same sample in a single assay) was 2.0% for $\langle r \rangle$.

Fluorescence lifetimes were determined with an Aminco-SLM 48000S apparatus (SLM-Aminco 48000S, SLM Instruments, Urbana, IL, USA), which works on the principle of phase and frequency modulation, using 20 frequencies within the range 8–180 MHz. A 450 W xenon lamp provides high energy to the excitation monochromator. The excitation wavelength was 365 nm (bandpass 8 nm) and a low wavelength cutoff filter (410 nm) was placed in the emission beam. The data were analyzed with the SLM 48000S software using a model of discrete exponential components (Lakowicz et al., 1984). A non-linear least squares routine was used and the reduced χ^2 test was used to measure goodness of the fit. All lifetime measurements were obtained using 9,10-diphenylanthracene in the reference cell. 9,10-diphenylanthracene in ethanol had a single exponential decay ($\tau = 9.24$ ns).

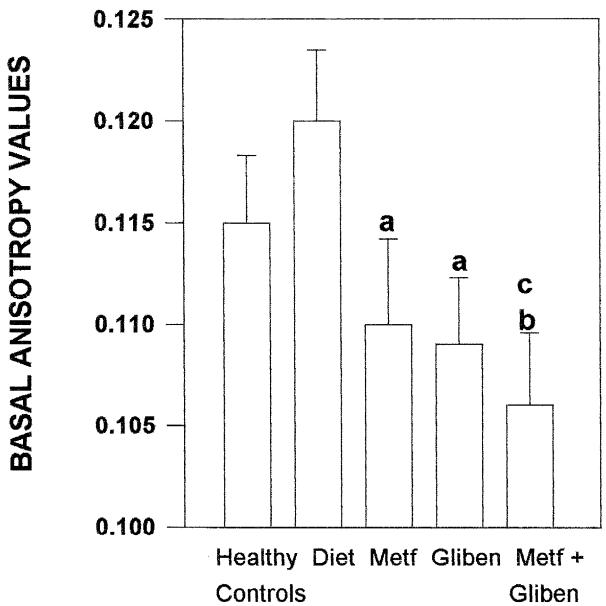
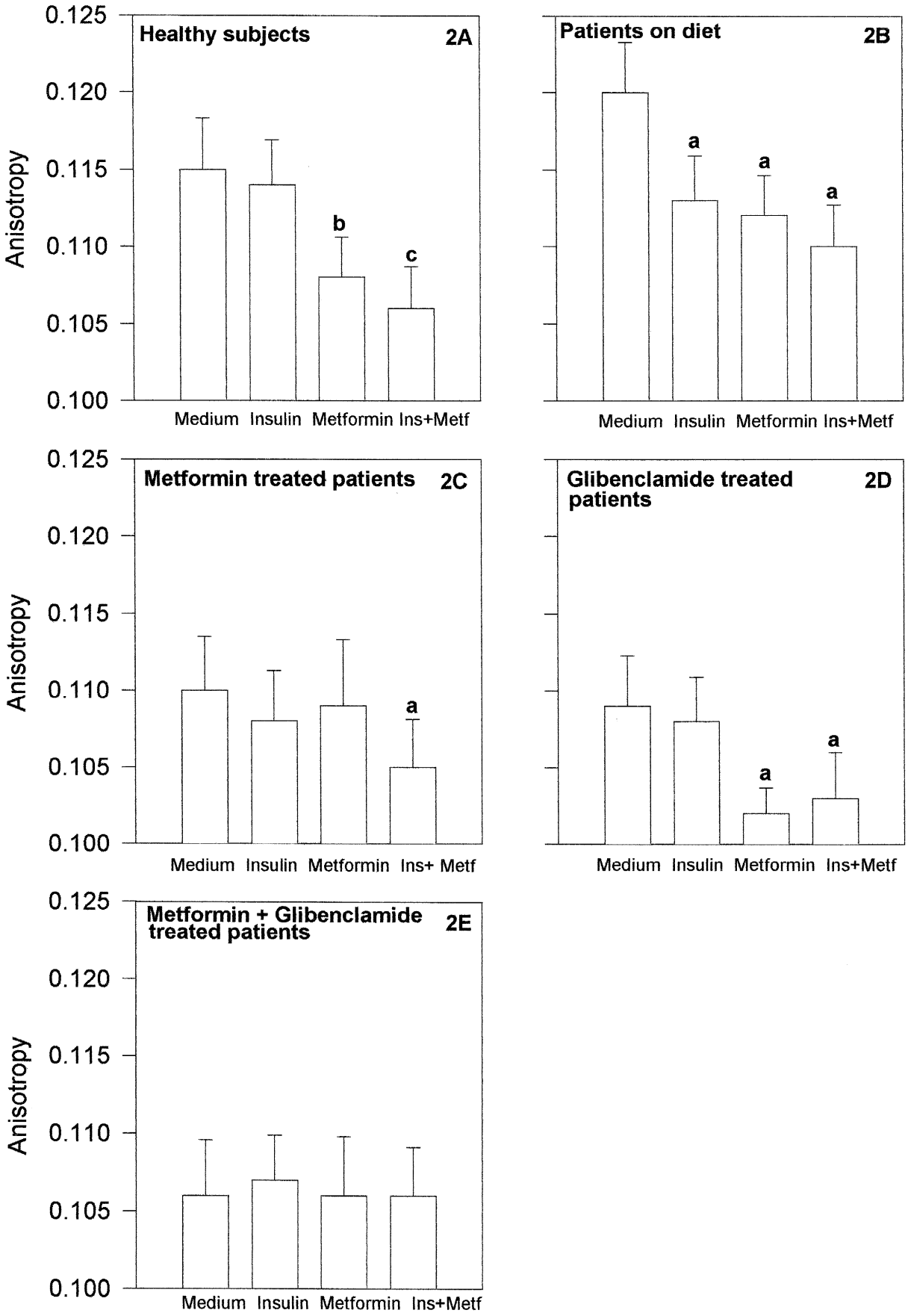


Fig. 1. Mean basal values ± S.E. of the fluorescence anisotropy of 6-anthroyloxystearic acid in erythrocytes from healthy controls and diabetic patients treated by diet, metformin (Metf), glibenclamide (Gliben) or metformin + glibenclamide (Metf + Gliben); [a] $P < 0.05$ and [b] $P < 0.01$ versus patients treated by diet; [c] $P < 0.05$ versus healthy controls.


Healthy subjects
2A
Patients on diet
2B
Metformin treated patients
2C
Glibenclamide treated patients
2D
Metformin + Glibenclamide treated patients
2E
Anisotropy
0.125
0.120
0.115
0.110
0.105
0.100
a
b
c
Medium
Insulin
Metformin
Ins+Metf
Ins+ Metf

2.8. Statistical analysis

Data are presented as means ± S.E. The distribution of variables was tested for normality. Significant differences between the healthy subjects and the four groups of patients were determined by one-way analysis of variance (ANOVA). Univariate statistical analysis was performed by linear regression analysis to indicate correlations between variables. The non-parametric paired Wilcoxon test was used to compare fluorescence anisotropy values before and after incubation with metformin and/or insulin. The unpaired Mann–Whitney test was used to compare the various groups. A probability of 5% or less was accepted as significant.

3. Results

The mean clinical parameters of the study groups are shown in Table 1. As expected, the healthy subjects and the diabetic patients significantly differ with respect to metabolic control parameters: fasting blood glucose and hemoglobin A1c. They differ also with respect to age and body mass index. Several subjects in the diabetic patients treated by diet or glibenclamide plus metformin were obese. The study groups did not differ with respect to plasma lipid parameters.

The anisotropy of the emitted fluorescence by the probe embedded in lipid areas of erythrocyte membrane is inversely related to membrane fluidity. The basal values for the groups differed (Fig. 1). The anisotropy of the probe in the erythrocyte membranes of patients on a restricted diet was higher than that recorded for the membranes of healthy controls, but the difference was not significant. The anisotropy values for the erythrocytes of patients treated with glibenclamide, metformin, or metformin plus glibenclamide were significantly lower than in the healthy controls and diet patients ($P < 0.05$ for the metformin or glibenclamide groups versus diet patients, $P < 0.05$ for metformin plus glibenclamide patients versus healthy controls and $P < 0.01$ for metformin plus glibenclamide patients versus diet). But the anisotropy values were not correlated with age, body mass index, fasting blood glucose, hemoglobin A1c. There also was no correlation between cholesterol, triglycerides, cholesterol/triglycerides ratio and fluorescence anisotropy.

The effect of incubating erythrocytes with insulin, metformin or the association insulin–metformin in vitro also differed depending on the group providing the erythrocytes. Incubating erythrocytes from healthy controls with insulin decreased the anisotropy marginally. Metformin had a greater effect; the anisotropy of the fluorescent probe decreased by about 7% ($P < 0.005$). Incubation with insulin plus metformin appeared to have an additive effect on the decrease in the anisotropy ($P < 0.001$) (Fig. 2A). The anisotropy of the probe on erythrocytes from diet-treated patients also decreased when they were incubated with insulin or metformin (Fig. 2B). Insulin significantly decreased the anisotropy of the probe embedded in the membrane of erythrocytes from diet-treated patients (6%, $P < 0.05$). Metformin reduced the anisotropy of the probe in erythrocytes from diet-treated patients to about the same extent as in healthy controls (7%, $P < 0.05$). Insulin plus metformin also decreased the anisotropy ($P < 0.05$). Erythrocytes from metformin-treated patients were not greatly influenced by metformin in vitro, with a very small decrease in probe anisotropy. But insulin plus metformin significantly decreased the anisotropy (5%, $P < 0.05$) (Fig. 2C). Insulin had little or no effect on erythrocytes from patients treated with glibenclamide but metformin and insulin plus metformin decreased the anisotropy (6%, $P < 0.05$) (Fig. 2D). Lastly, the erythrocytes from patients treated with metformin plus glibenclamide (Fig. 2E), showed no significant change in fluorescence anisotropy when incubated with insulin and/or metformin.

A direct interaction in vitro between insulin or metformin and the fluorescent probe might change the lifetime of the probe and hence induce artefactual changes in the anisotropy. We therefore measured the lifetime of 6-(9-anthroyloxy)stearic acid probe embedded in the membranes of erythrocytes incubated in vitro in absence or in presence of insulin or metformin. The lifetime of the probe in erythrocytes incubated without insulin or metformin was: 10.0 ± 0.4 ns. No significant change in the lifetime was recorded after the incubation of erythrocytes with insulin (9.8 ± 0.4 ns) or metformin (10.0 ± 0.6 ns). Therefore, there is no direct interaction between the biguanide or the hormone and the fluorescent probe. Thus, changes in the fluorescence anisotropy indeed reflect changes in the fluidity of the surroundings of the probe.

4. Discussion

This study investigates the interaction of insulin and metformin with erythrocyte membrane by analysing lipid fluidity using fluorescence polarization and 6-anthroyloxystearic acid as fluorescent probe. The lifetime of the fluorescent probe is not altered by incubating labelled erythrocytes with insulin and/or metformin. The results therefore reflect the actual effect of insulin and/or metformin on lipid fluidity and not any direct interaction of the drug or hormone with the probe.

Fig. 2. Effect of the in vitro incubation with insulin, metformin or insulin plus metformin on the fluorescence anisotropy of 6-anthroyloxystearic acid embedded in erythrocyte membranes from healthy subjects (A) and diabetic patients treated by diet (B), metformin (C), glibenclamide (D) and metformin plus glibenclamide (E); [a] $P < 0.05$, [b] $P < 0.005$, [c] $P < 0.001$ versus erythrocytes incubated in medium only.

The properties of erythrocytes from healthy subjects and patients with non-insulin dependent diabetes mellitus were examined, because the dynamic and structural properties of erythrocyte membranes from these patients are altered (Watala, 1993) and this could affect the interaction of insulin and metformin with membranes. Our data also show that the basal fluidity of erythrocyte membranes from healthy subjects and patients with non-insulin dependent diabetes mellitus is different, but this difference does not seem to be linked to the clinical, metabolic or lipid parameters. The treatment of patients could be responsible for the differences in basal fluidity. Published studies have shown an increase (Mazzanti et al., 1989), decrease (Kamada and Otsuji, 1983; Watala and Winocour, 1992) or no change (Caimi et al., 1988; Freyburger et al., 1988; Muzulu et al., 1994) in the membrane fluidity of erythrocytes of patients with non-insulin dependent diabetes mellitus. But it is difficult to compare these findings directly with our results, as data on treatments were limited. We find that the basal fluidity of erythrocyte membranes from diet-treated patients is lower than that of erythrocytes from healthy subjects, while the fluidity of erythrocytes from patients treated with metformin and/or glibenclamide is significantly higher. The high fluidity of the erythrocyte membranes from patients treated with glibenclamide is in agreement with Neufeld et al. (1987), who used the same spectroscopic method to show that treating diabetic patients with glyburide increased monocyte membrane fluidity. This is also the first time, to our knowledge, that the membrane fluidity of erythrocytes from non-insulin dependent diabetes mellitus patients treated with metformin has been shown to be elevated. The increased fluidity is similar to that of erythrocyte membranes from patients treated with metformin plus glibenclamide.

The in vitro incubation of erythrocytes with a supraphysiological dose of insulin shows that insulin in most cases increases membrane fluidity, in agreement with previous studies on erythrocytes (Bryszewska and Leyko, 1983; Dutta-Roy et al., 1985; Santini et al., 1992). A study on erythrocyte ghosts found no fluidizing action of insulin (Freisleben et al., 1992). These effects of insulin may differ because of the dose used (Dutta-Roy et al., 1985) and the experimental conditions. We find that insulin has a small fluidizing effect, except in the erythrocytes from patients treated by diet. The significant effect of insulin on the fluidity of the erythrocyte membrane from the patients treated by a hypocaloric diet could be related to the treatment. The amount and type of dietary fats affect the phospholipid fatty acid composition (Pagnan et al., 1989; Romon et al., 1995) and the phospholipid profile (Dougherty et al., 1987) of the membranes. Insulin receptor properties and insulin binding on erythrocyte membrane depend upon membrane lipid and fatty acids (Pelikánová et al., 1989; Maehara, 1991). Therefore, a higher fluidizing effect of insulin on the erythrocyte membranes of patients treated with diet might result from an insulin binding different from the binding on the erythrocytes of healthy subjects and other patients.

The results of the in vitro interaction of metformin with erythrocytes from healthy controls and diabetic subjects treated by diet or glibenclamide confirm the in vivo findings. Metformin has a fluidizing effect on erythrocyte membrane. But, it does not change in vitro the fluidity of erythrocytes from patients previously treated with metformin or glibenclamide plus metformin. This may be because metformin in the plasma of these patients has already interacted with their erythrocytes to increase basal fluidity. Using [^{14}C]metformin, it has been previously shown that the radioactivity cannot be entirely washed out from erythrocyte membranes (Freisleben et al., 1996). Therefore, despite the washings required in our study for sample preparation, it may be hypothesized that a small amount of metformin remains within the membrane. Hence, any further in vitro incubation with metformin could be unable to change further the membrane dynamics. The fact that the erythrocyte membranes of patients treated with glibenclamide have an elevated basal lipid fluidity and still significantly respond to metformin in vitro with a further increase in fluidity does not contradict the above explanation. Glibenclamide and metformin may affect different areas within the cell membrane.

The effects of the in vitro association insulin plus metformin on membrane fluidity depends on the group providing the erythrocytes. In the erythrocytes of healthy controls and patients treated by diet or metformin, the in vitro insulin metformin combination induced a higher fluidity than that of samples treated with insulin or metformin. But it is not clear if insulin potentiates the effect of metformin. The increase in membrane fluidity which is a membrane event common to both insulin and metformin supports the hypothesis that insulin and metformin could work additively. One question raised by the results of the metformin treated patients concerns our proposal that metformin from plasma is present within the erythrocyte membrane. Similar results in samples treated in vitro with insulin or insulin plus metformin could be expected. However, as suggested above, the concentration of metformin preexisting in the membrane is weak and does not correspond to the conditions of the in vitro insulin metformin combination. In contrast, the in vitro insulin metformin association does not change the fluidity of the erythrocytes of patients in vivo treated with glibenclamide plus metformin or glibenclamide, compared to the erythrocytes of the same patients incubated with metformin alone. One explanation is that the erythrocyte fluidity of these patients, either basal or resulting of the in vitro interaction with only metformin, is high. Therefore, the fluorescent probe can be unable to detect a further fluidizing effect after insulin addition.

In summary, metformin induces an increase in the fluidity of the membrane of intact erythrocytes both in vitro and in vivo. The in vitro findings are in agreement

with data obtained for isolated erythrocyte membranes (Freisleben et al., 1992). Our results suggest that metformin may act in vivo by increasing membrane lipid fluidity. Metformin increases the number of low affinity insulin receptors in erythrocytes (Holle et al., 1981; Rizkalla et al., 1986), probably as a consequence of the increased fluidity of the erythrocyte membrane. In obese women with normal glucose tolerance, metformin administration increases the number of insulin receptors and the tyrosine kinase activity per receptor of solubilised erythrocytes (Santos et al., 1997). However, erythrocytes are not insulin-sensitive and it is unlikely that metformin acts via insulin receptors in these cells. Several agents that alter membrane fluidity change glucose transport activity (Pilch et al., 1980; Yuli et al., 1981; Hutchinson et al., 1985). By normalizing membrane fluidity in diabetic conditions, metformin could enhance the efficacy of membrane-inserted glucose transporters by increasing their intrinsic activity (Yoa et al., 1993; Wiernsperger, 1996). Thus, any further increase in fluidity caused by insulin could enhance glucose transport activity. It is now necessary to examine the effect of metformin on the membrane fluidity of an insulin-sensitive cell like the adipocyte, to elucidate the contribution of membrane changes to the overall action of metformin.

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